

1                                    **Purification Means**

2  
3    The present invention relates to purification means,  
4    in particular to means suitable for use in  
5    purification of soluble proteins.

6

7    **Introduction**

8

9    The recombinant production of protein in bacteria,  
10   yeast, insect and mammalian cell lines has become a  
11   cornerstone of biological research and the  
12   biotechnology industry. Classical biochemical and  
13   chromatographical purification techniques usually  
14   produce inadequate amounts of a target protein to  
15   study its roles or actions. Even if enough of the  
16   protein can be purified, it usually involves  
17   cumbersome amounts of starting material or tissue  
18   and many processing steps are taken before  
19   reasonable purification can be achieved.

20

21   Recombinant expression of the target protein  
22   bypasses a lot of these problems. By introducing

1 the target protein's gene template to a cell line or  
2 bacterial culture, induced overexpression can result  
3 in significant levels of that protein being  
4 produced. Large amounts of protein make the  
5 purification a lot simpler, but the addition or  
6 fusion of purification domains or tags allows for a  
7 relatively simple one-step purification using  
8 affinity chromatography resins. However,  
9 occasionally, due to the varying nature of proteins,  
10 the production of soluble protein has remained  
11 elusive with known tags unable to purify many  
12 proteins. In some cases, production of protein can  
13 be a problem due to differences in the machinery of  
14 bacterial cells. There is therefore a need for a  
15 more versatile tag than is available currently on  
16 the market. The provision of such a versatile tag  
17 enabling, for example, improved ability to quickly  
18 produce and screen soluble protein in bacteria such  
19 as *E.coli* would represent a major step forward in  
20 protein biochemistry.

21

## 22 Summary of the Invention

23

24 The present inventors have developed a novel  
25 purification tag based on the gene product of a  
26 sortase gene, in particular the *srtA* gene of  
27 *Staphylococcus aureus*. This tag, known as SNUT  
28 [Solubility eNhancing Unique Tag] has been found to  
29 have exceptional activity, enabling the efficient  
30 purification of soluble domains of a number of  
31 proteins hitherto not able to be isolated  
32 efficiently using conventional purification tags.

1

2 Throughout this specification, reference to a SNUT  
3 Tag should be understood to mean a tag derived from  
4 a sortase gene product.

5

6 In a first aspect of the invention, there is  
7 provided a purification tag comprising a sortase,  
8 e.g srtA, gene product.

9

10 In preferred embodiments, the sortase gene product  
11 is a gene product of the srtA gene of *Staphylococcus*  
12 *aureus*.

13

14 Also provided is the use of a sortase, e.g srtA,  
15 gene product as a purification tag.

16

17 Furthermore, according to a third aspect of the  
18 invention, there is provided an expression construct  
19 for the production of recombinant polypeptides,  
20 which construct comprises an expression cassette  
21 consisting of the following elements that are  
22 operably linked: a) a promoter; b) the coding region  
23 of a DNA encoding a sortase, eg srtA gene product as  
24 a purification tag sequence; c) a cloning site for  
25 receiving the coding region for the recombinant  
26 polypeptide to be produced; and d) transcription  
27 termination signals.

28

29 According to a fourth aspect of the invention, there  
30 is provided a method for producing a polypeptide,  
31 comprising: a) preparing an expression vector for  
32 the polypeptide to be produced by cloning the coding

1 sequence for the polypeptide into the cloning site  
2 of an expression construct according to the third  
3 aspect of the invention; b) transforming a suitable  
4 host cell with the expression construct thus  
5 obtained; and c) culturing the host cell under  
6 conditions allowing expression of a fusion  
7 polypeptide consisting of the amino acid sequence of  
8 the purification tag with the amino acid sequence of  
9 the polypeptide to be expressed covalently linked  
10 thereto; and, optionally, d) isolating the fusion  
11 polypeptide from the host cell or the culture medium  
12 by means of binding the fusion polypeptide present  
13 therein through the amino acid sequence of the  
14 purification tag.

15

16 The expression construct, herein referred to as  
17 pSNUT, may be made by modification of any suitable  
18 vector to include the coding region of a DNA  
19 encoding a sortase. In preferred embodiments, the  
20 expression construct is based on the pQE30 plasmid.

21

22 A sample of pSNUT was deposited with the National  
23 Collections of Industrial and Marine Bacteria Ltd.  
24 (NCIMB), 23 St Machar Drive, Aberdeen, Scotland AB24  
25 3RY on 23 December 2002 under accession no NCIMB  
26 41153.

27

28 In a fifth aspect, there is provided a fusion  
29 polypeptide obtained by the method of the fourth  
30 aspect of the invention.

31

1 The inventors have found that when a fusion  
2 polypeptide comprising a polypeptide/protein of  
3 interest and a SNUT tag is used as an antigen, the  
4 immune response generated is significantly stronger  
5 than that generated when the polypeptide/protein of  
6 interest alone is used as the antigen.

7

8 Thus, in a sixth aspect of the present invention,  
9 there is provided a method of inducing and/or  
10 enhancing an immune response to an antigen of  
11 interest, the method comprising administering the  
12 antigen of interest with a sortase, e.g srtA, gene  
13 product. The antigen of interest, which preferably  
14 is a polypeptide/protein of interest, may be  
15 administered simultaneously, separately or  
16 sequentially with the sortase, e.g srtA, gene  
17 product. In preferred embodiments, the antigen of  
18 interest is linked to the sortase, e.g srtA, gene  
19 product, preferably in the form of a fusion  
20 polypeptide.

21

22 In a seventh aspect of the invention, there is  
23 provided the use of a sortase, e.g srtA, gene  
24 product as an immunogen. As with the sixth aspect,  
25 the sortase, e.g srtA, gene product is preferably  
26 administered as a fusion polypeptide comprising the  
27 sortase, e.g srtA, gene product and an antigen of  
28 interest.

29

30 In preferred embodiments, the sortase, e.g. srtA  
31 gene product (SNUT) is encoded by the nucleotide  
32 sequence shown in Figure 4 or a variant or fragment

1    thereof. Preferably, the srtA gene product  
2    comprises amino acids 26 to 171 of the SrtA sequence  
3    shown in Figure 4 or a variant or fragment thereof.

4  
5    Variants and fragments of and for use in the  
6    invention preferably retain the functional  
7    capability of the polypeptide i.e. ability to be  
8    used as a purification tag. Such variants and  
9    fragments which retain the function of the natural  
10   polypeptides can be prepared according to methods  
11   for altering polypeptide sequence known to one of  
12   ordinary skill in the art such as are found in  
13   references which compile such methods, e.g.  
14   Molecular Cloning: A Laboratory Manual, J. Sambrook,  
15   et al., eds., Second Edition, Cold Spring Harbor  
16   Laboratory Press, Cold Spring Harbor, New York,  
17   1989, or Current Protocols in Molecular Biology, F.  
18   M. Ausubel, et al., eds., John Wiley & Sons, Inc.,  
19   New York.

20  
21   A variant nucleic acid molecule shares homology  
22   with, or is identical to, all or part of the coding  
23   sequence discussed above. Generally, variants may  
24   encode, or be used to isolate or amplify nucleic  
25   acids which encode, polypeptides which are capable  
26   of ability to be used as a purification tag.

27  
28   Variants of the present invention can be artificial  
29   nucleic acids (i. e. containing sequences which have  
30   not originated naturally) which can be prepared by  
31   the skilled person in the light of the present  
32   disclosure. Alternatively they may be novel,

1 naturally occurring, nucleic acids, which may be  
2 isolatable using the sequences of the present  
3 invention. Thus a variant may be a distinctive part  
4 or fragment (however produced) corresponding to a  
5 portion of the sequence provided in Figure 4. The  
6 fragments may encode particular functional parts of  
7 the polypeptide.

8  
9 The fragments may have utility in probing for, or  
10 amplifying, the sequence provided or closely related  
11 ones.

12  
13 Sequence variants which occur naturally may include  
14 alleles or other homologues (which may include  
15 polymorphisms or mutations at one or more bases).  
16 Artificial variants (derivatives) may be prepared by  
17 those skilled in the art, for instance by site  
18 directed or random mutagenesis, or by direct  
19 synthesis. Preferably the variant nucleic acid is  
20 generated either directly or indirectly (e. g. via  
21 one or amplification or replication steps) from an  
22 original nucleic acid having all or part of the  
23 sequences of Figure 4. Preferably it encodes a  
24 polypeptide which can be used as a purification tag.

25  
26 The term 'variant' nucleic acid as used herein  
27 encompasses all of these possibilities. When used in  
28 the context of polypeptides or proteins it indicates  
29 the encoded expression product of the variant  
30 nucleic acid.

31

1 Homology (i. e. similarity or identity) may be as  
2 defined using sequence comparisons are made using  
3 FASTA and FASTP (see Pearson & Lipman, 1988. Methods  
4 in Enzymology 183 : 6398). Parameters are preferably  
5 set, using the default matrix, as follows :  
6 Gapopen (penalty for the first residue in a gap) :-  
7 12 for proteins/-16 for DNA  
8 Gapext (penalty for additional residues in a gap) :-  
9 2 for proteins/-4 for DNA  
10 KTUP word length : 2 for proteins/6 for DNA.  
11 Homology may be at the nucleotide sequence and/or  
12 encoded amino acid sequence level. Preferably, the  
13 nucleic acid and/or amino acid sequence shares at  
14 least about 60%, or 70%, or 80% homology, most  
15 preferably at least about 90%, 95%, 96%, 97%, 98% or  
16 99% homology with the sequence shown in Figure 4.  
17  
18 Thus a variant polypeptide in accordance with the  
19 present invention may include within the sequence  
20 shown in Figure 4, a single amino acid change or 2,  
21 3, 4, 5, 6, 7, 8, or 9 changes, or about 10, 15, 20,  
22 30, 40 or 50 changes. In addition to one or more  
23 changes within the amino acid sequence shown, a  
24 variant polypeptide may include additional amino  
25 acids at the C terminus and/or N-terminus.  
26  
27 Naturally, regarding nucleic acid variants, changes  
28 to the nucleic acid which make no difference to the  
29 encoded polypeptide (i.e. 'degeneratively  
30 equivalent') are included within the scope of the  
31 present invention.  
32



1 Preferred variants include one or more of the  
2 following changes(using the annotation of AF162687):  
3 nucleotide 604 AAG causing an amino acid mutation of  
4 KAR; nucleotide 647 AAG, codon remains K, therefore  
5 a silent mutation; nucleotide 982 GAA causing an  
6 amino acid mutation of GAE.

7  
8 Changes to a sequence, to produce a derivative, may  
9 be by one or more of addition, insertion, deletion  
10 or substitution of one or more nucleotides in the  
11 nucleic acid, leading to the addition, insertion,  
12 deletion or substitution of one or more amino acids  
13 in the encoded polypeptide. Changes may be by way of  
14 conservative variation, i. e. substitution of one  
15 hydrophobic residue such as isoleucine, valine,  
16 leucine or methionine for another, or the  
17 substitution of one polar residue for another, such  
18 as arginine for lysine, glutamic for aspartic acid,  
19 or glutamine for asparagine. As is well known to  
20 those skilled in the art, altering the primary  
21 structure of a polypeptide by a conservative  
22 substitution may not significantly alter the  
23 activity of that peptide because the side-chain of  
24 the amino acid which is inserted into the sequence  
25 may be able to form similar bonds and contacts as  
26 the side chain of the amino acid which has been  
27 substituted out. This is so even when the  
28 substitution is in a region which is critical in  
29 determining the peptides conformation.

30

31 Also included are variants having non-conservative  
32 substitutions. As is well known to those skilled in

1 the art, substitutions to regions of a peptide which  
2 are not critical in determining its conformation may  
3 not greatly affect its activity because they do not  
4 greatly alter the peptide's three dimensional  
5 structure.

6  
7 In regions which are critical in determining the  
8 peptides conformation or activity such changes may  
9 confer advantageous properties on the polypeptide.  
10 Indeed, changes such as those described above may  
11 confer slightly advantageous properties on the  
12 peptide e. g. altered stability or specificity.

13  
14 SNUT tags and vectors may be used in methods of  
15 purifying a soluble domain of a peptide.  
16 Accordingly in a further aspect of the invention,  
17 there is provided a method of producing a soluble  
18 bioactive domain of a protein, the method  
19 comprising the steps of cloning DNA encoding at  
20 least one candidate soluble domain into at least one  
21 expression vector, transfecting or transforming a  
22 host cell with said vector, expressing said DNA in  
23 said host cell, wherein said vector encodes a  
24 sortase gene product.

25  
26 The sortase gene product is preferably in the form  
27 of a fusion protein.

28  
29 The method may comprise the steps of analysis of DNA  
30 coding for the protein of interest to identify  
31 antigenic soluble domains, designing oligonucleotide  
32 primers to amplify DNA encoding the domain,

1    amplifying DNA, cloning the DNA, optionally  
2    screening clones for correct orientation of DNA,  
3    expressing DNA in expression strains, analysing  
4    expression products for solubility, analysing  
5    products and production of soluble bioactive protein  
6    domain.

7

8    The method optionally comprises the step of  
9    producing a soluble bioactive protein domain of said  
10   protein of interest.

11

12   The methods and tags of the invention may be used  
13   with any suitable polypeptide/protein of interest,  
14   for example for the purification of such  
15   polypeptides/proteins of interest. As described  
16   herein and exemplified in the following examples,  
17   the inventors have demonstrated that the methods and  
18   tags of the invention enable the efficient  
19   purification of a a large number of proteins, many  
20   of which have not been amenable to efficient  
21   isolation using conventional methods and tags.

22

23   In preferred embodiments of the invention, the  
24   polypeptide/protein of interest is MAR1, Jak1 or  
25   CD33, or a fragment thereof.

26

27   In particularly preferred embodiments, the  
28   polypeptide/protein of interest is a variable domain  
29   fragment e.g. a variable domain fragment of CD33.

30

1 Preferred features of each aspect of the invention  
2 are as for each of the other aspects mutatis  
3 mutandis.

4

5 The invention is exemplified with reference to the  
6 following non limiting description and the  
7 accompanying figures in which:

8

9 Figure 1 shows selected domains for amplification  
10 from *in silico* analysis. Representation of a  
11 candidate protein for the expression platform, in  
12 this case Jak1 (human). Four fragments have been  
13 chosen by analysis as depicted.

14

15 Figure 2 shows denaturing dot-blot analysis of  
16 expression clones of fragments of MAR1 in pQE30.

17

18 Figure 3 shows a ribbon Diagram of *Staphylococcus*  
19 *aureus* sortase. Ribbon diagram of the putative  
20 structure of *S. aureus* SrtA protein (minus its N-  
21 terminal membrane anchor). SNUT represents the  
22 portion of this structure between the two yellow  
23 arrows as shown. The yellow ball signifies a  $\text{Ca}^{2+}$   
24 ion, essential for the biological activity of this  
25 protein. This diagram is taken from Hlangovan et  
26 al., 2001, PNAS 98 (11) 6056  
27 (doi:10.1073/pnas.101064198)

28

29 Figure 4 shows the Nucleotide Sequence and amino  
30 acid sequence of SNUT fragment.

31

1 (a) This is the determined sequence of SNUT. The  
2 fragment was cloned into pQE30 using the BamHI site  
3 of this vector. When in the wanted orientation,  
4 insertion results in the inactivation of the  
5 upstream cloning site, therefore allowing any  
6 subsequent cloning of target inserts with the  
7 downstream BamHI site (see (b) for restriction map  
8 of sequence).

9  
10 Figure 5 illustrates qualitative purification  
11 results using the SNUT fusion tag. (a) shows the  
12 elution profile on SDS-PAGE of SNUT-Jak1 using AKTA  
13 Prime native histag purification. Successful  
14 elution of SNUT-Jak1 construct is signified by the  
15 white arrow. (b) shows the elution profile on SDS-  
16 PAGE of SNUT-MAR1 using AKTA Prime native histag  
17 purification. Successful elution is shown by the  
18 arrow. (c) shows the same gel stained in (b)  
19 western blotted and detected using poly-histidine-  
20 HRP antibody. This is confirmation that the eluted  
21 species in (b) is actually SNUT-MAR1, of expected  
22 molecular weight.

23  
24 Figure 6 shows a Western blot of lysates using anti-  
25 histag antibody.

26  
27 Figure 7a illustrates the elution profile on SDS-  
28 PAGE of SNUT-CD33.

29  
30 Figure 7b illustrates a Western blot of the same gel  
31 from Figure 7a using anti-histag antibody to detect  
32 the proteins.

1 Figure 8a illustrates a Western blot using anti-  
2 histag antibody to detect the proteins.

3

4 Figure 8b illustrates a Western blot of the same gel  
5 as Figure 8b using anti-SrtA antibody to detect the  
6 proteins.

7

8 Figure 8C shows a Western blot showing the detection  
9 of the SNUT protein using an anti-SrtA monoclonal  
10 antibody.

11

#### 12 Template analysis and primer design

13

14 Analysis of the DNA coding for a protein of interest  
15 may be performed using software packages such as  
16 Vector NTI (Informax, USA) and  
17 BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>), p-fam (  
18 [www.sanger.ac.uk/pfam](http://www.sanger.ac.uk/pfam)) and TM pred  
19 ([www.hgmp.mrc.ac.uk](http://www.hgmp.mrc.ac.uk)) which may be used to identify  
20 complete domains within the protein that  
21 significantly increase the likelihood of  
22 antigenicity and/or solubility when expressed as a  
23 subunit of the original protein coding sequence.

24

25 In order to increase the possibility of identifying  
26 a soluble domain, preferably multiple sub-domains,  
27 more preferably at least three sub-domains, for  
28 example 3 to 9 sub-domains may be identified for  
29 processing.

30

31 Oligonucleotide primers to amplify the selected sub-  
32 domains may be designed with the help of

1 commercially available software packages such as the  
2 internet software package Primer3 ([http://www-](http://www-genome.wi.mit.edu/genome_software/other/primer3.html)  
3 [genome.wi.mit.edu/genome\\_software/other/primer3.html](http://www-genome.wi.mit.edu/genome_software/other/primer3.html)  
4 (Whitehead Institute for Biomedical Research),  
5 Vector NTI ([www.informaxinc.com](http://www.informaxinc.com)) and DNASIS (Hitachi  
6 Software Engineering Company ([www.oligo.net](http://www.oligo.net))).

7  
8 Typically primers for use in a method of the  
9 invention are in the range 10-50 base pairs in  
10 length, preferably 15 to 30, for example 20 base  
11 pairs in length, with annealing temperatures in the  
12 range 45-72°C, more conveniently 55-60°C. Primers  
13 may be synthesised using standard techniques or may  
14 be sourced from commercial suppliers such as  
15 Invitrogen Life Technologies (Scotland) or MWG-  
16 Biotech AG (Germany).

17

#### 18 PCR of Insert

19

20 The desired inserts which encode the selected sub-  
21 domains are amplified using the primers designed  
22 specifically for that target gene using standard PCR  
23 techniques. The template DNA for amplification can  
24 be in the form of plasmid DNA, cDNA or genomic DNA,  
25 depending on whatever is appropriate or indeed  
26 available. Any suitable DNA polymerase may be used,  
27 for example, Platinum Taq, Pfu ([www.stratagene.com](http://www.stratagene.com))  
28 or Pfx ([www.invitrogen.com](http://www.invitrogen.com)). Any suitable PCR system  
29 may be used, for example, the Expand High Fidelity  
30 PCR system (Roche, Basel, Switzerland).

31

1 Several different thermocycler conditions may be  
2 used with each set of primers. This increases the  
3 chance of the PCR working without having to  
4 individually optimise each new primer set. Typically  
5 the following three programs may be used in the  
6 method:

7

- 8 1. A standard PCR programme using the recommended  
9 annealing temperature provided with the  
10 primers.
- 11 2. A standard PCR programme using 50°C as the  
12 temperature for annealing.
- 13 3. A touchdown PCR programme, where the annealing  
14 temperature starts at a high temperature e.g  
15 65°C for 10 cycles and then gradually decreases  
16 the annealing temperature to 50°C over the  
17 subsequent e.g 15 cycles.

18

19 Buffer conditions may be adjusted as required, for  
20 example with respect to magnesium ion concentration  
21 or addition of DMSO for the amplification of  
22 difficult templates. Further details of a suitable  
23 purification method which may be used with the  
24 vector or tag of the invention can be found in our  
25 co-pending PCT application PCT/GB02/05941, filed on  
26 the same day as this application, published 24 July  
27 2003, and claiming priority from GB 0131026.7.

28

29 The PCR products may be visualised using standard  
30 techniques, for example on a 1.5% agarose gel  
31 stained with Ethidium Bromide and the bands are cut



1 out of the gel and purified using Mini elute gel  
2 extraction Kit (Qiagen, Crawley, England).

3

#### 4 Expression Vectors

5

6 Amplified DNA inserts may be cloned into expression  
7 vectors using techniques dictated by the multiple  
8 cloning sites of the vector in question. Such  
9 techniques are readily available to the skilled  
10 person.

11

12 Any suitable expression system can be used in the  
13 invention. Preferably, the expression system is  
14 prokaryotic. Suitable vectors for use in the method  
15 of the invention include any vector which can encode  
16 SNUT [Solubility eNhancing Unique Tag], for example  
17 pSNUT. This tag is based on the sequence of a trans-  
18 peptidase found on the surface of gram-positive  
19 bacteria. This protein is highly soluble, and  
20 expressed as very high levels.

21

22 The inventors have found that SNUT is an ideal  
23 fusion tag for conferring solubility and expression  
24 levels to target protein fragments. SNUT may be  
25 cloned into any suitable vector. For the purposes of  
26 the examples shown in this application, the sequence  
27 incorporating the SNUT fragment is cloned into pQE30  
28 (Qiagen, Valencia, CA) in a manner allowing full use  
29 of the multiple cloning site (MCS) of this vector  
30 for downstream gene insertions.

31

1    Development of pSNUT

2

3    The inventors found that a tag based on the *srtA*  
4    gene product from *Staphylococcus aureus* is highly  
5    soluble, reacts well to purification schemes and  
6    expresses particularly well. It was hypothesised  
7    that the incorporation of a portion or domain of  
8    this protein could represent a useful fusion tag in  
9    the present method, and indeed the expression of any  
10   poorly soluble protein in *E. coli*. Using NMR  
11   studies, the 3D structure of this protein has been  
12   predicted and is shown in Figure 3. We hypothesised  
13   that by taking a portion of this structure, we could  
14   make a manipulateable protein tag, but not disturb  
15   its tertiary structure enough to reduce its highly  
16   favourable characteristics listed above. The region  
17   of this protein used as a solubility-enhancing tag  
18   is depicted by two arrows.

19

20   The SNUT tag was cloned into pQE30. However, it may  
21   be cloned into any suitable expression vector.  
22   Positive clones may be identified by denaturing dot  
23   blots, SDS-PAGE and Western blotting. Final  
24   confirmation of these clones was provided by DNA  
25   sequencing, and the sequence of the multiple cloning  
26   region of the resultant vector is shown in Figure 4.

27

28   Variances in the sequence of the SNUT domain were  
29   observed from the sequence for *SrtA* that has been  
30   logged in Genbank (AF162687). The variances are  
31   (using the annotation of AF162687) nucleotide 604  
32   AAG causing an amino acid mutation of KAR;

1 nucleotide 647 AAG, codon remains K, therefore a  
2 silent mutation; nucleotide 982 GAA causing an amino  
3 acid mutation of GAE.

4  
5 Preliminary trials and native purification showed  
6 that the SNUT fragment was very soluble and its  
7 characteristics were in no way diminished by  
8 truncation, thus showing that SNUT could represent a  
9 useful tag domain (data not shown). As described in  
10 the Examples, to fully test the abilities of SNUT,  
11 we then chose two proteins were soluble protein  
12 production had proved impossible using conventional  
13 methods and using the other expression systems of  
14 the method of the present invention. Surprisingly,  
15 we found that, using pSNUT in the method of the  
16 invention, these proteins could be produced in  
17 soluble form.

18

#### 19 Clone Propagation

20

21 Target insert/expression vector ligations may be  
22 propagated using standard transformation techniques  
23 including the use of chemically competent cells or  
24 electro-competent cells. The choice of the host  
25 cell and strain for transformation is dependent on  
26 the characteristics of the expression vectors being  
27 utilised.

28

29 Bacterial cells, for example, *Escherichia coli*, are  
30 the preferred host cells. However, any suitable  
31 host cell may be used. In preferred embodiments, the  
32 host cells are *Escherichia coli*.

1  
2 The vectors may be used to each transfect or  
3 transform a plurality of different host cell  
4 strains. The set of host cell strains for  
5 individual vector may be the same or different from  
6 the set used with other vectors.

7  
8 In a particularly preferred embodiment of the  
9 invention, each vector may be transformed into three  
10 *E. coli* strains (for example, selected from  
11 Rosetta(DE3)pLacI, Tuner(DE3)pLacI, Origami BL21  
12 (DE3)pLacI and TOP10F, Qiagen).

13  
14 Where the vectors are pQE based vectors, TOP10F'  
15 cells are preferred for the propagation and  
16 expression trials of such vectors. The present  
17 inventors have identified this strain as a more  
18 superior strain for these vectors than either of the  
19 recommended strains by the supplier (M15 and  
20 SG13009), in terms of ease of use and culture  
21 maintenance (only one antibiotic required as to two  
22 with M15 or SG13009 ([www.qiagen.com](http://www.qiagen.com))). Other F'  
23 strains such as XL1 Blue can be used, but are  
24 inferior to the TOP10F' strain, due to lack of  
25 expression regulation (results not shown). The use  
26 of TOP10F' (Invitrogen) for the propagation and/or  
27 expression pQE based vectors forms an independent  
28 aspect of the present invention. Other F' strains  
29 such as XL1 Blue may also be used, but are inferior  
30 to the TOP10F'.

31

1 After transformation, cells may be plated out onto  
2 selection plates and propagated for the development  
3 of single colonies using standard conditions.

4

#### 5 Propagation of Cells

6

7 The colonies may be used to inoculate duplicate  
8 wells in a 96 well plate.

9

10 Typically, each well may contain 200  $\mu$ l of LB broth  
11 with the appropriate antibiotics. Each plate may be  
12 dedicated to one strain of *E. coli* or other host  
13 cell which alleviates the problems of different  
14 growth rates. The necessary controls are also  
15 included on each plate. The plates are then grown  
16 up, preferably at 37°C or any other temperature as  
17 appropriate to the particular host cell and vector,  
18 with shaking, until log phase is reached. This is  
19 the primary plate.

20

21 From the primary plate a secondary plate is seeded  
22 and then grown. Typically, the secondary plate is  
23 be seeded using 'hedgehog' replicators and then  
24 grown up to, for example, log phase, chilled to 16°C  
25 for 1 hour. Determination of positive clones from  
26 these plates may be undertaken using functional  
27 studies. Routinely, 6-48 clones for each insert-  
28 vector ligation are taken and propagated in culture  
29 micro-titre plates containing up to 500  $\mu$ l of media.  
30 According to the conditions and reagents required,  
31 protein production is then induced, and cultures  
32 propagated further. Most vectors are under the

1 control of a promoter such as T7, T7lac or T5, and  
2 can be easily induced with IPTG during log phase  
3 growth. Typically, cultures are propagated in a  
4 peptone-based media such as LB or 2YT supplemented  
5 with the relevant antibiotic selection marker.  
6 These cultures are grown at temperatures ranging  
7 from 4-40 °C, but more frequently in the range of  
8 20-37 °C depending on the nature of the expressed  
9 protein, with or without shaking and induced when  
10 appropriate with the inducing agent (usually log or  
11 early stationary phase). After induction, growth  
12 propagation can be continued for 1-16 hours for a  
13 detectable amount of protein to be produced.

14

15 The primary plate is preferably stored at 4°C until  
16 the process is complete.

17

18 Colony Screening for Inserts in Correct Orientation

1 The method of the invention may include the step of  
2 testing transformants for correct orientation of the  
3 inserts. Identification of positive clones can be  
4 achieved through a variety of methods, including  
5 standard techniques such as digestion analysis of  
6 plasmid DNA; colony PCR and DNA sequencing.  
7 Alternatively, dot-blotting may be used for the  
8 identification of positive clones for example, using  
9 a BioDot apparatus (BioRad) containing  
10 nitrocellulose membrane (0.45µm pore size) in  
11 accordance with the manufacturers' instructions,  
12 prior to final confirmation by DNA sequencing.

13,  
14 The use of this dot blotting method in the platform  
15 represents a rapid, reproducible and robust  
16 detection method. This particular method is useful  
17 for the rapid detection or presence of recombinant  
18 protein and allows for a determination of all clones  
19 irrespective of solubility and conformation. This  
20 may be important at this stage, because  
21 conformational structures can inhibit the detection  
22 of tag domains if they are not presented properly on  
23 the surface of the protein. This can occur as  
24 easily with both soluble and insoluble protein.

25  
26 As described above, standard colony PCR techniques  
27 may be used. For example, transformants may be  
28 selected, either manually or using automation such  
29 as the Cambridge BioRobotics BioPick instrument, and  
30 screened using directional PCR using a primer that  
31 encodes for a sequence on the vector such as S Tag  
32 or GATA sequence, and then the complementary primer

1 from the insert. A PCR mix may be used such as the  
2 RedTaq DNA Polymerase (Sigma Aldrich, Dorset,  
3 England) and the thermocycler conditions used may be  
4 the standard PCR programme using 50°C as the  
5 annealing temperature or adjusted as required.

6  
7 Although all colony selecting and picking can be  
8 done manually, automated colony pickers are  
9 preferred. Automated colony pickers such as the  
10 BioRobotics BioPick allow for the uniform and  
11 reproducible selection of clones from transformation  
12 plates. Clone selection determinants can be set to  
13 ensure picking colonies of a standardised size and  
14 shape. After picking and plate inoculation,  
15 propagation of clones can be carried out as  
16 described above.

17  
18 Identification of positive clones can be achieved  
19 through a variety of methods, including standard  
20 techniques such as digestion analysis of plasmid  
21 DNA; colony PCR and DNA sequencing. Alternatively, in  
22 a preferred embodiment, the novel method of dot-  
23 blotting described herein for the identification of  
24 positive clones may be used in place of such  
25 traditional techniques, prior to final confirmation  
26 by DNA sequencing. The use of this method in the  
27 platform presented here is not essential in the use  
28 of this platform over existing screening  
29 methodologies, but represents a rapid, reproducible  
30 and robust detection method. The protocol described  
31 here is a new protocol for an existing method for



1 which commercially available equipment (Bio-Rad  
2 DotBlot) can be purchased.

3  
4 This particular method is useful for the rapid  
5 detection or presence of recombinant protein and  
6 allows for a determination of all clones  
7 irrespective of solubility and conformation. This  
8 is useful at this stage, because conformational  
9 structures can inhibit the detection of tag domains  
10 if they are not presented properly on the surface of  
11 the protein. This can occur as easily with both  
12 soluble and insoluble protein.

13  
14 For example, after growth on the micro-titre plates  
15 is complete, the plate is centrifuged at 4000 rpm  
16 for 10 minutes at 4°C to harvest the bacterial  
17 cells. The supernatant is removed and the cell  
18 pellets are re-suspended in 50 µl lysis buffer (10  
19 mM Tris.HCl, pH 9.0, 1mM EDTA, 6 mM MgCl<sub>2</sub>)  
20 containing benzonase (1 µl/ml). The plate is  
21 subsequently incubated at 4°C with shaking for 30  
22 minutes. A sample (10 µl) of the cell lysate is  
23 added to 100 µl buffer (8 M urea, 500 mM NaCl, 20 mM  
24 sodium phosphate, pH 8.0) and incubated at room  
25 temperature for 20 minutes. Samples are then  
26 applied to a BioDot apparatus (BioRad) containing  
27 nitrocellulose membrane (0.45µM pore size) in  
28 accordance with the manufacturers' instructions.  
29 The membrane is removed and transferred into  
30 blocking reagent (3% w/v; Bovine serum albumin in  
31 TBS) for 30 minutes at room temperature. The blot  
32 is washed briefly with TBS then incubated in a

1 primary antibody, specific to the tag being used for  
2 the subset of expression clones. Depending on the  
3 nature of the primary i.e., whether or not it has a  
4 horse radish peroxidase (HRP) reporter function,  
5 will depend on whether the use of a secondary is  
6 required. For detection of specific binding the  
7 membrane is then washed 2x 5 minutes in TBS followed  
8 by 1x 5 minute wash in 10 mM Tris.HCl pH7.6.  
9 Detection of specifically bound antibody is  
10 disclosed by the addition of chromogenic substrate  
11 (6 mg diaminobenzidine in 10 ml 10 mM Tris.HCl pH  
12 7.6 containing 50  $\mu$ l 6% H<sub>2</sub>O<sub>2</sub>) . The reaction is  
13 stopped by thorough rinsing in water. Positive  
14 clones identified by this procedure can then be  
15 confirmed by DNA sequencing of the expression  
16 construct using now industry-standard techniques and  
17 equipment such as ABI and Amersham Biosciences.

#### 19 Sequencing

21 The sequencing reactions may be performed using  
22 techniques common in the art using any suitable  
23 apparatus. For example, sequencing may be performed  
24 on the cloned inserts, using the Big Dye Terminator  
25 cycle sequencing kits (Applied Biosystems,  
26 Warrington, UK) and the specific sequencing primer  
27 run on a Peltier Thermal cycler model PTC225 (MJ  
28 Research Cambridge, Mass). The reactions may be run  
29 on Applied Biosystems - Hitachi 3310 Sequencer  
30 according to the manufacturer's instructions. These  
31 sequences are checked to ensure that no PCR  
32 generated errors have occurred.

1

2   **Assessment of Solubility of Positive Clones**

3

4   The cells of positive clones may be harvested and  
5   soluble and insoluble protein detected.

6

7   Any suitable techniques known in the art can be used  
8   to separate soluble and insoluble protein, such as  
9   the use of centrifugation, magnetic bead  
10   technologies and vacuum manifold filtrations.

11   Typically, however, the separated proteins are  
12   ultimately analysed by acrylamide gel and western  
13   blotting. This confirms the presence of recombinant  
14   protein at the correct size.

15

16   In one embodiment, contents of each well in the 96  
17   well plate are transferred into a Millipore 0.65  $\mu$ m  
18   multi-screen plate. The plate is placed on a vacuum  
19   manifold and a vacuum is applied. This draws off  
20   the culture medium to waste. The cells are then  
21   washed with PBS (optional), again the vacuum is  
22   applied to remove the PBS. The multi-screen plate is  
23   removed from the manifold and bacterial cell lysis  
24   buffer (containing DNase) (50  $\mu$ l) is added to each  
25   well. The plate is incubated at room temperature  
26   for 30 minutes with shaking to facilitate lysis of  
27   the cells. A fresh 96 well microtitre plate (ELISA  
28   grade) is placed inside the vacuum manifold and the  
29   multi-screen plate is placed above it. When a  
30   vacuum is applied the contents of each well are  
31   drawn into the micro-titre plate below. The vacuum  
32   only needs to be applied for 20 seconds. The

1 collected lysate contains the soluble fraction of  
2 expressed protein. A sample of the collected lysate  
3 may subsequently analysed by SDS-PAGE and Western  
4 blotting to confirm both the presence and correct  
5 molecular weight of the target protein.

6  
7 The use of SDS-PAGE and Western blotting can be  
8 expensive and time consuming, especially when  
9 numerous samples must be analysed for each  
10 construct. In light of this we have developed a  
11 protocol whereby one gel can be used for both total  
12 protein staining and western blotting. This  
13 represents a significant improvement in this  
14 methodology and obviously allows cost saving, and  
15 precise comparisons can be made with regard to total  
16 protein and western blotting as both sets of results  
17 come from the one gel.

18  
19 The basis of this protocol is in the ability to use  
20 chloroform and UV light to stain protein on an SDS-  
21 PAGE gel (Kazmin et al., Anal Biochem, 2001, 301(1)  
22 91-6; doi:10.1006/abio.2001.5488). We have used  
23 this technique to great effect as it allows for the  
24 extremely rapid staining of a SDS-PAGE gel in less  
25 than a tenth of the time taken using other more  
26 traditional staining methods such as Commassie  
27 Brilliant Blue and Collodial Blue stains. We then  
28 decided to take this observation a step further and  
29 analyse the ability of a chloroform-stained gel to  
30 be used in Western blotting. This would not be  
31 expected to work as other stained gels result in the  
32 fixing of the protein to the gel and subsequent

1 inability to transfer the protein during blotting.  
2 This expectation is coupled to the fact that  
3 chloroform is not compatible with western blotting  
4 equipment (Bio-Rad SD blotter user's manual).  
5 However, fortuitously, we have discovered that with  
6 a wash of the chloroform-stained gel in double-  
7 distilled water, to remove excess chloroform, and  
8 after subsequent soaking in transfer buffer,  
9 proteins were effectively transferred during western  
10 blotting in contrast to expectations. This transfer  
11 was no-less effective than from a gel that has not  
12 been pre-stained with chloroform and UV light.  
13 Figure 6 primarily shows results relating to the  
14 production of soluble protein by the platform, but  
15 also shows the ability to use the chloroform-stained  
16 SDS-PAGE derived western blot for the identification  
17 of proteins, without any apparent damage caused to  
18 the proteins.

19

20 The use of a chloroform-stained SDS-PAGE derived  
21 western blot for the identification of proteins  
22 forms another aspect of the present invention.

23

#### 24 **Scale-Up and Purification**

25

26 This analysis provides a picture of the expression  
27 status of the clones on each plate. Using this  
28 analysis, positive soluble protein expressing clones  
29 can be identified for the production of soluble  
30 recombinant protein for a given target protein. The  
31 clones may be selected and their growth scaled up  
32 e.g. to 5 ml scale, using the saved primary plate as

1 an inoculum. Parameters that may be taken into  
2 consideration in deciding on the appropriate culture  
3 to select for scale-up include the desirability of  
4 specific regions for the production of an antigen,  
5 the overall expression levels of the clone and  
6 factors that may affect affinity purification such  
7 as amino acid composition.

8

9

## 10 Examples

11

### 12 Example 1. Expression construct design

13

14 Figure 1 is a diagrammatic representation of the  
15 protein Jak1. Using pfam, the position of distinct  
16 domains was established. Further analysis of these  
17 domains was then carried out using Tmpred and the  
18 Kyle and Dolittle hydrophobicity algorithm to  
19 determine the usefulness of these domains as soluble  
20 antigens. From this tentative analysis, four  
21 domains were selected for amplification and  
22 expression analysis. Based on this preliminary *in*  
23 *silico* analysis, primers specific for a target  
24 protein were designed and used to amplify domains  
25 selected for analysis.

26

27 Vectors (500 ng) were restricted with *Bam*HI (20  
28 units) and *Sal*I (20 units) in the presence of calf  
29 intestinal alkaline phosphatase (CIP) (2 units), gel  
30 purified and quantified using standard methods.  
31 Purified PCR fragments (100 ng) were restricted with  
32 *Bam*HI (5 units) and *Sal*I 5 units), gel purified,

1 quantified, and then used in a ligation reaction  
2 with the restricted vector again using standard T4  
3 DNA ligase methods (Ready-to-Go T4 DNA ligase,  
4 Amersham Biosciences). A sample of the ligation  
5 reaction (1  $\mu$ l) was then used to transform the  
6 appropriate competent bacterial cells (TOP10F' were  
7 used here for the pQE based vectors, a modification  
8 of the manufacturers recommendations; BL21(DE3)pLyse  
9 for pET43.1a and TOP10F' for pGEX-Fus).  
10 Transformants were selected on LB/ampicillin (100  
11  $\mu$ g/ml) overnight at 28°C.

12  
13 A Cambridge BioRobotics BioPick instrument was used  
14 for the picking of 24 colonies from each of the  
15 transformant plates into flat-bottomed and lidded  
16 micro-titre plates. The clones were used to  
17 inoculate 150  $\mu$ l of LB (containing 100 $\mu$ g/ml  
18 ampicillin), and these were allowed to grow  
19 overnight at 37 °C.

20  
21 A secondary plate was prepared by the inoculation of  
22 200  $\mu$ l of LB containing the required supplements  
23 with 10  $\mu$ l of the overnight primary culture. These  
24 were then grown at 37 °C Once an optical density  
25 (OD) of 0.25 at A550 was reached, IPTG (final  
26 concentration, 1 mM) was added to induce expression  
27 of the recombinant protein. Culture propagation was  
28 continued for another 4 hours prior to harvesting of  
29 bacterial cells.

30  
31 After clones expressing specific recombinant protein  
32 have been identified, the solubility of these

1 proteins has to be established prior to clone  
2 selection for purification. This can be performed a  
3 number of ways including the use of centrifugation  
4 and automation-friendly vacuum manifold separations.  
5 The results here were obtained using methodologies  
6 based around the use of vacuum-assisted filtration  
7 to separate soluble and insoluble protein. The  
8 filtrates that were produced from the method  
9 described were then analysed by SDS-PAGE and Western  
10 blotting to confirm the production of a recombinant  
11 protein of the correct anticipated molecular weight.  
12

#### 13 Example 2 Design and Construction of SNUT Expression 14 Tag

15  
16 Based on analysis of the amino acid sequence and  
17 predicted structure of SrtA<sub>AN</sub>, it was decided to  
18 amplify the region of amino acids 26 to 171 of the  
19 SrtA sequence. Amplification was conducted using  
20 the forward primer 5' TTTTGTAGATCTAAACCACATATCGAT  
21 and the reverse primer 5'  
22 TTTTGTGGATCCATCTAGAACTTCTAC. This product was then  
23 digested with *Bgl*I and *Bam*HI and ligated into pQE30  
24 vector which had also been digested with *Bam*HI to  
25 form the pSNUT vector. The ligation mix was  
26 transformed into TOP10F' cells and single colonies  
27 propagated on LB agar containing 100 µg/ml  
28 ampicillin. Clones with the *srtA* fragment in the  
29 correct orientation were screened by expression  
30 analysis and positive clones identified using the  
31 denaturing dot-blot assay described earlier.



1 The sequence encoding the SNUT tag was cloned into  
2 pQE30 as described earlier and positive clones  
3 identified by denaturing dot blots, SDS-PAGE and  
4 Western blotting. Final confirmation of these  
5 clones was provided by DNA sequencing, and the  
6 sequence of the multiple cloning region of the  
7 resultant vector is shown in Figure 4. Variances in  
8 the sequence of the SNUT domain were observed from  
9 the sequence for SrtA that has been logged in  
10 Genbank (AF162687). The variances are (using the  
11 annotation of AF162687) nucleotide 604 AAG causing  
12 an amino acid mutation of KAR; nucleotide 647 AAG,  
13 codon remains K, therefore a silent mutation;  
14 nucleotide 982 GAA causing an amino acid mutation of  
15 GAE.

16

### 17 **Example 3 Trials of SNUT Expression Constructs**

18

19 Target inserts were cloned into the pSNUT vector  
20 using primer construction and digestion of resulting  
21 PCR amplifications with *Bam*HI and *Sal*I as described  
22 earlier. pSNUT was digested with *Bam*HI in a similar  
23 manner and the target inserts cloned as described.  
24 Clones were screened using the denaturing dot-blot  
25 system and then analysed with SDS-PAGE and western  
26 blotting. Positive clones were used for preparative  
27 200 ml LB cultures containing 100 µg/ml ampicillin  
28 and induced as described earlier. This was grown to  
29 an optical density of 0.5 at  $A_{550}$  at 37 °C.  
30 Expression of SNUT was then induced with the  
31 addition of IPTG (final concentration, 1 mM) and

1 left to grow for another 4 hours. Cells were then  
2 harvested by centrifugation at 5K rpm for 15  
3 minutes. Cells were re-suspended in 30 ml PBS  
4 containing 0.1% Igepal and lysis induced by two  
5 freeze-thaw cycles. The suspension was then  
6 sonicated and centrifuged at 5K rpm for 15 minutes.  
7 The soluble supernatant was transferred to a fresh  
8 container and filtered through a 0.8 µm disc filter  
9 to remove final cell debris. This solution was then  
10 applied to a Ni<sup>2+</sup> charged IMAC column (Amersham  
11 Biosciences HiTrap Chelating column, 1 ml) using an  
12 AKTA Prime low pressure chromatography system and  
13 column was then treated using a standard native his-  
14 tag purification protocol involving washing of  
15 column with 20 mM sodium dihydrogen phosphate pH 8.0  
16 containing 10 mM imidazole, 500 mM NaCl, and elution  
17 of soluble his-tagged proteins using 20 mM sodium  
18 dihydrogen phosphate pH 8.0 containing 500 mM  
19 imidazole, 500 mM NaCl. Elution fractions were then  
20 analysed on an SDS-PAGE gel (4-20% SDS-PAGE Bio-Rad  
21 Criterion gel), which was stained with chloroform as  
22 described earlier. This gel was then subsequently  
23 western blotted and the his-tagged protein detected  
24 with anti-poly-histidine monoclonal antibody using  
25 the techniques described herein.

26  
27 Preliminary trials and native purification showed  
28 that the SNUT fragment was very soluble and its  
29 characteristics were in no way diminished by  
30 truncation, thus showing that SNUT could represent a  
31 useful tag domain (data not shown). To fully test  
32 the abilities of SNUT, we then chose two proteins

1 for which soluble protein production had proved  
2 impossible using the other expression systems in  
3 which SNUT was not used as a tag. These were murine  
4 MAR1 and human Jak1. Clones were prepared and  
5 selected using the method as described in the  
6 Examples above and positive clones were subsequently  
7 grown and induced at 37 °C. These were then treated  
8 to identical native histag purifications. Both  
9 proteins behaved very favourably under standard  
10 purification conditions as can be seen from the  
11 purification profiles in Figure 5. For both these  
12 trial proteins, this was the first example of such  
13 purification under soluble conditions. The  
14 production of these proteins using conventional  
15 techniques has failed to produce any soluble  
16 protein, irrespective of expression system or growth  
17 conditions used (data not shown). However, as  
18 described in this example, when the protein  
19 fragments were expressed in pSNUT, soluble proteins  
20 can be surprisingly obtained.

21  
22 The effectiveness of SNUT as a fusion protein is  
23 even more significant when it is considered that no  
24 special growth conditions were required for the  
25 generation of soluble protein. This is remarkable  
26 when one considers the protein expressionist's  
27 standard GST tag which is not even soluble itself  
28 when expressed at 37 °C; 28 °C is required before  
29 even the generation of GST on its own without any  
30 target protein is observed.

31

1    **Example 4 Purification of CD33 fragments using SNUT**  
2    **Expression Constructs**

3  
4    **Cloning Results**

5  
6    CD33 contains two extracellular immunoglobulin  
7    domains. The extracellular region of the CD33 DNA  
8    sequence had been cloned into several vectors for  
9    expression, including expression as a fusion tag to  
10    DHFR and NusA. None of these vectors produced  
11    recombinant CD33 protein. The CD33 extracellular  
12    region was also cloned into pSNUT. Both pSNUT and  
13    CD33 were restricted with BamH1 and HindIII under  
14    standard conditions and ligated together using T4  
15    DNA ligase, again under standard manufacturer's  
16    protocols. TOP10F' cells were transformed with the  
17    ligation product.

18  
19    6 colonies were picked from the transformation plate  
20    and grown in 150µl LB in a 96-well plate at 37°C  
21    overnight

22  
23    **Expression analysis:**

24  
25    The overnight cultures were used to inoculate fresh  
26    LB cultures (10µl into 190µl LB + 50µg/ml  
27    ampicillin) and grown at 37°C for 2 hours.

28    Expression of the SNUT-CD33 construct was induced  
29    with 1mM IPTG.

30  
31    Cells were pelleted after 4 hours and lysed in PBS +  
32    0.1% Igepal. Lysates were analysed by western blot

1 using anti-histag antibody. As shown in Figure 6,  
2 it was clear that colonies 1, 3 and 4 were positive  
3 and 2 was not (SNUT only).

4

#### 5 Large Scale Expression:

6

7 The clone pertaining to lane 1 of Figure 6 was  
8 chosen for sequencing analysis, which proved  
9 successful insertion into the pSNUT vector. This  
10 clone was grown in large scale (200ml) for  
11 expression of the SNUT-CD33 construct at 37°C.  
12 Expression was induced whenever the OD600=0.4-0.6.  
13 After 4-6 hours expression, the cells were pelleted  
14 and lysed in 8M urea buffer. Lysates were clarified  
15 and purified by immobilised metal affinity  
16 chromatography (IMAC) using a re-folding technique  
17 of decreasing urea concentration. At 0M urea, the  
18 SNUT-CD33 was eluted from the IMAC column and  
19 analysed by SDS PAGE using Coomassie blue stain  
20 (Figure 7A) and Western Blotting (Figure 7B) using  
21 anti-histag antibody.

22

#### 23 Antibody Detection of expressed protein:

24

25 The SNUT fusion protein contains an N-terminal His-  
26 tag. This facilitates detection using commercially  
27 available anti-His antibodies, and can be used as a  
28 means for purification of the recombinant protein  
29 via IMAC as described (see Figure 8a).

30

31 In addition, we have developed in-house a polyclonal  
32 antibody against SNUT and it also provides a

1 detection and purification means, as demonstrated in  
2 Figure 8b.

3

4 Furthermore, the inventor has developed monoclonal  
5 antibodies against SNUT which may also be used in  
6 detection and purification methods of the invention.  
7 A hybridoma producing monoclonal antibodies against  
8 SNUT was developed as follows:

9

10 4 BALB/c mice were immunised intraperitoneally with  
11 a purified SNUT recombinant protein. Seven  
12 inoculations of 50µl of the antigen mixed with 50µl  
13 of adjuvant were given over a ten-week time course.  
14 Test bleeds were taken at intervals and positive  
15 immunisation was confirmed by Western blot. Two days  
16 after final inoculation, the mouse spleen cells were  
17 fused with SP2 myeloma cells. The resulting  
18 hybridoma cells were maintained in HAT media.  
19 Microtitre plates were coated with the immunising  
20 antigen (50ng/well) together with a control. Eleven  
21 days post fusion actively growing Hybridoma cells  
22 were ELISA screened for specificity to SNUT. Those  
23 giving high readings were cloned twice by limiting  
24 dilutions. An ECL of supernatant was performed as a  
25 final control of their specificity.

26

27 Figure 8C shows a Western blot showing the detection  
28 of the SNUT protein using one of the monoclonal  
29 antibodies developed.

30

31 Results:

32

1 CD33 has been a very difficult protein to express.  
2 The most desirable part of the protein for antigen  
3 production is the extracellular variable domain.  
4 There are two immunoglobulin domains in the  
5 extracellular region of CD33, a membrane distal  
6 variable (IgV) domain and a membrane proximal  
7 constant (C2) domain. Expression analysis had been  
8 performed for three fragments of the extracellular  
9 region: the variable domain, the constant domain and  
10 the full extracellular region in a number of  
11 commercially available expression vectors. Only the  
12 constant domain fragment would express in any of the  
13 vectors. In order to express the desired variable  
14 domain, the full length extracellular fragment and  
15 the IgV domain fragment were cloned into our pSNUT  
16 vector. Expression was successful for the full  
17 length fragment.

18  
19 The full length fragment was also purified  
20 successfully by re-folding on an IMAC column. Not  
21 only has the pSNUT vector allowed us to express a  
22 protein fragment that has been unable to be  
23 expressed in any tried commercially available  
24 vector, including vectors with fusion tags designed  
25 to increase expression such as NusA and DHFR, but  
26 has allowed us to purify the expressed protein using  
27 immobilised metal affinity chromatography by  
28 standard techniques, and can be used for detection  
29 of any protein expressed in the vector using either  
30 anti-His or anti-SrtA antibodies.

31

1 All documents referred to in this specification are  
2 herein incorporated by reference. Various  
3 modifications and variations to the described  
4 embodiments of the inventions will be apparent to  
5 those skilled in the art without departing from the  
6 scope and spirit of the invention. Although the  
7 invention has been described in connection with  
8 specific preferred embodiments, it should be  
9 understood that the invention as claimed should not  
10 be unduly limited to such specific embodiments.  
11 Indeed, various modifications of the described modes  
12 of carrying out the invention which are obvious to  
13 those skilled in the art are intended to be covered  
14 by the present invention.  
15